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Pestivirus NS3 (p80) Protein Possesses RNA Helicase Activity

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The pestivirus bovine viral diarrhea virus (BVDV) p80 protein (referred to here as the NS3 protein) contains amino acid sequence motifs predictive of three enzymatic activities: serine proteinase, nucleoside triphosphatase, and RNA helicase. We have previously demonstrated that the former two enzymatic activities are associated with this protein. Here, we show that a purified recombinant BVDV NS3 protein derived from baculovirus-infected insect cells possesses RNA helicase activity. BVDV NS3 RNA helicase activity was specifically inhibited by monoclonal antibodies to the p80 protein. The activity was dependent on the presence of nucleoside triphosphate and divalent cation, with a preference for ATP and Mn^{2+}. Hydrolysis of the nucleoside triphosphate was necessary for strand displacement. The helicase activity required substrates with an un-base-paired region on the template strand 3′ of the duplex region. As few as three un-base-paired nucleotides were sufficient for efficient oligonucleotide displacement. However, the enzyme did not act on substrates having a single-stranded region only to the 5′ end of the duplex or on substrates lacking single-stranded regions altogether (blunt-ended duplex substrates), suggesting that the directionality of the BVDV RNA helicase was 3′ to 5′ with respect to the template strand. The BVDV helicase activity was able to displace both RNA and DNA oligonucleotides from RNA template strands but was unable to release oligonucleotides from DNA templates. The possible role of this activity in pestivirus replication is discussed.

The *Pestivirus* genus of the family *Flaviviridae* is composed of three viruses, bovine viral diarrhea virus (BVDV), border disease virus, and classical swine fever virus. All are economically important disease agents in their respective animal species. Like viruses from the other two genera of the *Flaviviridae* (7), the flaviviruses and hepatitis C viruses, pestiviruses contain a positive-sense, single-stranded RNA genome containing a single large open reading frame (ORF), which in their case is about 12,500 nucleotides (nt) in length and encodes approximately 4,000 amino acids (4). The complete protein complement of pestiviruses has been established. The gene product order along the ORF is NH_2-[nonstructural protein p20-capsid protein p14-envelope glycoproteins gp48, gp25, gp55-nonstructural proteins p125 (p54/p80), p10, p32, p58, p75]-COOH (4–6, 22, 35).

Toward understanding the functional roles of these pestivirus proteins in virus replication, a number of enzymatic activities have been shown, or predicted, to be associated with pestivirus polypeptides. The first product of the ORF, the p20 protein, acts as a proteinase to autocatalytically release itself from the polyprotein precursor (35, 40). The p20 protein is a nonstructural protein unique to pestiviruses; a homologous polypeptide is not found in the flaviviruses and hepatitis C viruses. Another unique pestivirus protein is the first viral envelope glycoprotein (gp48). This structural protein possesses an associated RNase activity (15, 31). Roles for these two proteins and their respective enzymatic activities in the pestivirus life cycle remain to be established.

On the basis of the presence of conserved amino acid sequence motifs, two additional pestivirus proteins have been predicted to possess enzymatic activities. In both cases, the pestivirus proteins have homologous counterparts in the flaviviruses and hepatitis C viruses. One of these is the carboxy-terminal protein product in the ORF of these viruses, the pestivirus p75 protein, the flavivirus NS5 protein, and the hepatitis C virus NS5B protein. Each possesses sequence motifs characteristic of RNA-directed RNA polymerases (16, 25). Whereas these polypeptides are likely the respective viral polymerases, to date there are no direct biochemical data confirming this prediction.

The second of the proteins with comparable enzymatic function among members of this virus family is the NS3 protein (or p80 protein in the case of pestiviruses). In fact, this polypeptide is multifunctional. Amino acid sequence motifs predictive of serine proteinase, nucleoside triphosphatase (NTPase), and RNA helicase activities all reside within the same polypeptide chain (2, 8–10, 19). For representative viruses from each genus in the *Flaviviridae*, the serine proteinase activity has been experimentally demonstrated (1, 3, 11, 12, 26, 36, 41). Similarly, the NS3 proteins have all been shown to exhibit an RNA-stimulated NTPase activity (33, 34, 37, 39). Here, we demonstrate that the p80 protein of the pestivirus BVDV (to be referred to herein as the BVDV NS3 protein) possesses the third predicted enzymatic activity, RNA helicase activity. Our initial description of the properties of this pestivirus RNA helicase activity is the subject of this report. We have also established that the NS3 proteins of the yellow fever flavivirus and of hepatitis C virus possess a similar activity. These data will be presented elsewhere.

MATERIALS AND METHODS

Expression and purification of the BVDV NS3 protein. The recombinant baculovirus bacD2-133 has been previously described (24), as has the immunoaffinity purification of the p80 (NS3) protein from lysates of bacD2-133-infected *Spodoptera frugiperda* Sf9 cells (34).

Preparation of helicase substrates. Helicase substrates consisted of two complementary nucleic acid strands that were annealed and gel purified. The larger of the two strands was unlabeled and is referred to as the template strand. The smaller strand, referred to as the release strand, was ^{32}P-labeled. RNA strands were prepared by in vitro transcription of commercially available plasmids. Cold

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and [α-^{32}P]CTP-containing transcription reaction mixtures utilized SP6 or T7 RNA polymerase (Promega) and were carried out according to the manufacturer's recommendations. DNA strands used in helicase substrates were either chemically synthesized or purified by denaturing polyacrylamide gel electrophoresis. Several series of substrates were prepared and are schematically depicted in Fig. 1, 6, and 7. The details of their preparation follow.

(i) Standard substrate. *Bst*NI-digested plasmid pSP65 (Promega) was transcribed with SP6 polymerase to produce a 154-nt template strand. *Bam*HI-digested plasmid pSP64 (Promega) was transcribed with SP6 polymerase in the presence of [α-^{32}P]CTP to produce the radiolabeled 34-nt release strand. Transcription reactions were treated with RQ1 DNase (Promega) and then were extracted with phenol-chloroform and precipitated with ethanol. Transcripts were combined at a molar ratio of radiolabeled release strand to unlabeled template strand of approximately 10:1 in 0.5 M NaCl–25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-HCl (pH 7.4)–1 mM EDTA–0.1% sodium dodecyl sulfate (SDS). The mixture was heated for 5 min at 95°C and 30 min at 55°C and then incubated overnight at 25°C. Hybridization reactions were ethanol precipitated with 20 μg of glycogen carrier, resuspended in RNA sample buffer (20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.02% Triton X-100, 0.1% SDS, 10% glycerol, 0.02% bromophenol blue), and electrophoresed on an 8% polyacrylamide (acryl-bis, 30:0.8)–0.5× TBE (BRL Gibco)–0.1% SDS gel. The duplex substrate band was localized by autoradiography. The gel slice was excised, pulverized, and extracted with 0.5 M NH_4 acetate–2 mM EDTA–0.1% SDS for 2 h at 37°C. Eluted substrate was ethanol precipitated and resuspended in TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA).

(ii) 3′/3′ substrate. Plasmid pGEM3CS$^-$ (29) was digested with *Rsa*I and transcribed with T7 RNA polymerase to produce a 103-nt template strand. The same plasmid was digested with *Pvu*II and transcribed with SP6 polymerase in the presence of [α-^{32}P]CTP to produce a radiolabeled 57-nt release strand. The two transcripts were annealed and gel purified as described above.

(iii) 5′/5′ substrate. Plasmid pSP73.H64 was constructed by ligation of the 64-bp *Hae*III-*Hae*III fragment from pSP65 into the *Pvu*II site of pSP73. Digestion with *Bam*HI and transcription with T7 RNA polymerase produced a 118-nt template strand. The same plasmid was digested with *Hin*dIII and transcribed with SP6 polymerase in the presence of [α-^{32}P]CTP to produce a radiolabeled 75-nt release strand. The two transcripts were annealed and gel purified as described above.

(iv) Blunt substrate. A portion (2 μg) of the gel-purified standard substrate described above was incubated at 30°C in 20 μl of nuclease buffer (50 mM sodium acetate [pH 5.0], 30 mM NaCl, 1 mM $ZnSO_4$) containing 30 U of mung bean nuclease (New England Biolabs). After 15 min, the reaction was terminated by the addition of RNA sample buffer. The resulting 29-nt blunt-ended duplex substrate was gel purified as described above.

(v) R/D* substrates. A 34-nt DNA oligonucleotide whose sequence corresponds to the deoxyribonucleotide version of the standard substrate release strand described above was synthesized and end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). This radiolabeled DNA release strand was annealed with the standard substrate RNA template strand to yield the RNA/DNA (R/D*) helicase substrate (Fig. 1). Additional RNA transcript/DNA oligonucleotide substrates of various structures (see Fig. 6 and 7) were similarly prepared.

(vi) DNA/RNA (D/R*) and DNA/DNA (D/D*) substrates. To prepare a DNA template strand, the 144-bp *Eco*RI-*Bst*NI fragment from plasmid pSP65 was heated in formamide sample buffer (90% formamide, 1× TBE, 0.02% bromophenol blue) and electrophoresed on an 8% polyacrylamide (acryl-bis, 20:1)–7 M urea–1× TBE sequencing gel. Localization of the separated DNA strands was aided by running the same fragment that had been end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase in adjacent tracks. After autoradiographic localization of the labeled marker strands, the region of the gel containing the 144-nt unlabeled single-stranded DNA corresponding to nucleotides 11 to 154 of the pSP65-*Bst*NI-SP6 polymerase transcript was excised and extracted. This single-stranded DNA template strand was annealed with either the radiolabeled standard substrate RNA release strand (for D/R*) or with the end-labeled 34-nt DNA oligonucleotide release strand corresponding to this transcript (for D/D*).

Helicase assays. Standard helicase reactions (10 μl) were carried out as follows, unless otherwise specified in the text. NS3 protein (0.1 pmol) and approximately 3,000 cpm (0.16 to 0.32 pmol) of helicase substrate were added to a mixture having a final constitution of 25 mM MOPS (morpholinepropanesulfonic acid)-KOH (pH 6.5), 5 mM ATP, 3 mM $MnCl_2$, 2 mM dithiothreitol, 100 μg of bovine serum albumin per ml, and 5 U of RNasin (Promega). Reactions were incubated 30 min at 37°C and terminated by the addition of 2.5 μl of 5× RNA sample buffer (100 mM Tris-HCl (pH 7.5), 50 mM EDTA, 0.1% Triton X-100, 0.5% SDS, 50% glycerol, 0.1% bromophenol blue). Mixtures were electrophoresed on 15% polyacrylamide (acryl-bis, 30:0.8)–0.5× TBE–0.1% SDS gels at 15 mA of constant current until bromophenol blue dye approached the bottom of the gel. Gels were dried and exposed to X-ray film. Helicase activity was quantified by cutting the substrate and released strand bands from the dried gels and measuring radioactivity.

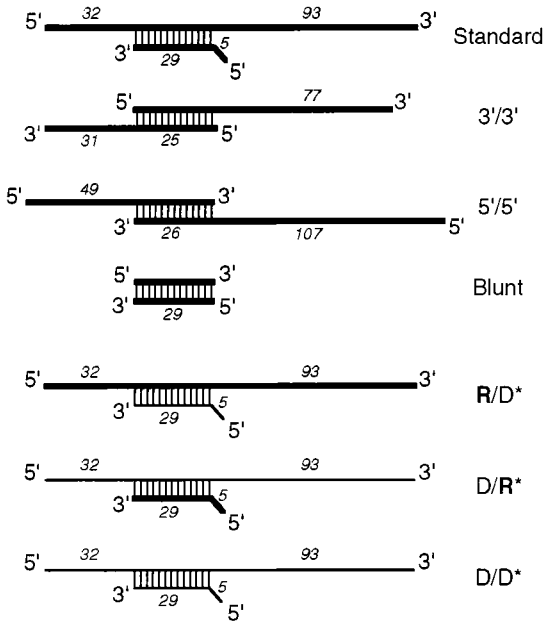

FIG. 1. Schematic representation of helicase substrates. Preparation and composition of each substrate is described in Materials and Methods. Thick lines represent RNA strands (R), thin lines depict DNA strands (D), and vertical lines represent regions of base pairing. For the DNA-containing substrates, asterisks denote the radiolabeled release strand. The italicized numbers indicate nucleotide lengths in that portion of the substrate.

RESULTS

Detection of RNA helicase activity associated with purified recombinant pestivirus NS3 protein. We have previously described the engineered expression and subsequent immunoaffinity purification of a recombinant form of the BVDV NS3 (p80) protein produced by a recombinant baculovirus in Sf9 insect cells (24, 34). We showed that the purified NS3 preparations possessed a specific RNA-stimulated NTPase activity, whereas preparations derived from NS3-deficient lysates did not; that the NS3 protein was the only polypeptide in these preparations to bind ATP; and that the NTPase activity was inhibited by anti-NS3 monoclonal antibodies but not by unrelated antibodies (34). Here, using the same enzyme preparations, we evaluated their ability to effect the strand displacement of duplex nucleic acid substrates. We initially constructed a standard helicase substrate, consisting of an RNA template strand of 154 nt annealed with a shorter radiolabeled RNA release strand of 34 nt, 29 nt of which were complementary to the template strand (Fig. 1). Incubation of this substrate in a helicase reaction mixture containing the pestivirus NS3 protein, followed by gel electrophoresis, showed the release of the faster migrating, radiolabeled 34-nt strand from the duplex substrate (Fig. 2, lane 3). Strand dissociation was dependent on the presence in the reaction mixture of divalent cation, since omission of $MnCl_2$ (lane 4) or addition of EDTA to the complete reaction mixture (not shown) eliminated the release of the radiolabeled 34-nt RNA strand from the duplex substrate. ATP was also required for helicase activity (lane 5). Moreover, hydrolysis of ATP was necessary for activity, since substitution of ATP with a nonhydrolyzable ATP analog (adenosine 5′-*O*-(3-thiotriphosphate [ATPγS]) eliminated strand displacement (lane 6). The fact that the observed helicase activity was due to the BVDV NS3 polypeptide was demonstrated by its requirement in the complete reaction mixture (lane 2) and by inhibi-

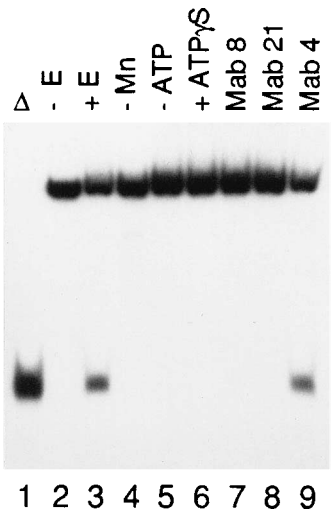

FIG. 2. RNA helicase activity in a purified preparation of BVDV NS3 protein. The NS3 protein of BVDV was purified by immunoaffinity chromatography as previously described (34). Using standard reaction conditions (except as noted below) and standard substrate, helicase activity was assessed by gel electrophoresis followed by autoradiography. Lanes 1 and 2 represent reaction mixtures lacking enzyme that were either boiled (Δ) or left native (− E). Lane 3, complete reaction mixture. Lanes 4, 5, and 6, complete reaction mixtures with $MnCl_2$ omitted (lane 4), with ATP omitted (lane 5), or with the nonhydrolyzable analog ATPγS substituted for ATP (lane 6). Lanes 7, 8, and 9, NS3 enzyme preparation preincubated at 4°C for 30 min with 2 μg of either p80-specific murine monoclonal antibody (Mab) 8.12.7 (lane 7) or 21.5.8 (lane 8) (6a) or with a murine monoclonal antibody specific for an antigen unrelated to BVDV proteins (lane 9) (4D4, anti-Rift Valley fever virus G2 glycoprotein [16a]) prior to being added to the complete reaction mixtures.

tion of the activity by monoclonal antibodies specific to the p80 protein (lanes 7 and 8) but not by an unrelated monoclonal antibody (lane 9).

Optimal reaction conditions for the BVDV NS3 helicase activity. The above biochemical data serve to demonstrate that the recombinant BVDV NS3 protein does indeed possess the RNA helicase activity that had been predicted from the presence of particular amino acid sequence motifs (10, 19). We next determined the optimal reaction conditions for the BVDV NS3 helicase activity, using the standard duplex RNA substrate. As shown in Fig. 2, the helicase activity required divalent cation and ATP. However, we found that the ratio of these two components was important for optimal strand displacement. Figure 3 shows the RNA helicase activity at several ratios of Mg^{2+} to ATP (panel A) or Mn^{2+} to ATP (panel B). With both divalent cations, the greatest helicase activity was observed at 3 mM divalent cation–5 mM ATP, the highest ATP concentration tested. Mn^{2+} was preferred over Mg^{2+} under all conditions tested. At the optimal concentration of Mn^{2+} and ATP, the pH optimum for the reaction was 6.5 (panel C). Under all conditions tested, the addition of monovalent cations (NaCl or KCl) to reaction mixtures inhibited helicase activity (data not shown).

We tested the ability of nucleoside triphosphates (NTPs) other than ATP to support the BVDV NS3 RNA helicase activity. With the standard substrate, the enzyme was able to utilize all of the common NTPs, albeit at efficiencies that were three to eight times less than the activity supported by ATP (Table 1).

Preferred structures of RNA helicase substrates. All of the data above were obtained by using the standard duplex RNA substrate which contained both 5′ and 3′ single-stranded RNA regions or tails (Fig. 1). To determine if the BVDV NS3 RNA

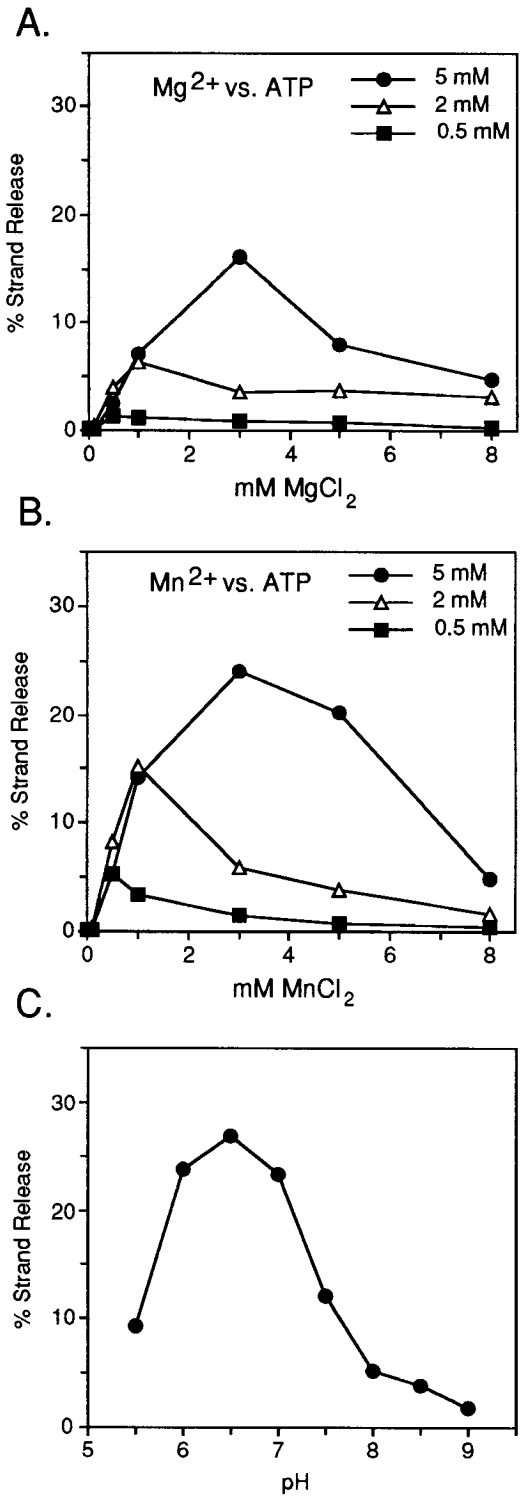

FIG. 3. Optimization of BVDV NS3 RNA helicase reaction conditions. Using the standard RNA substrate, helicase reactions were conducted and the activity was quantified, all as described in Materials and Methods. (A) $MgCl_2$ (3 mM) at three different ATP concentrations. (B) $MnCl_2$ (3 mM) at three different ATP concentrations. (C) The optimal pH for the RNA helicase activity was determined in 25 mM buffer at the indicated pH in 3 mM $MnCl_2$–5 mM ATP. Buffers were as follows: pH 5.5 and 6.0, 2-(*N*-morpholino)ethanesulfonic acid (MES)-HCl; pH 6.5 and 7.0, MOPS-KOH; pH 7.5-9.0, Tris-HCl.

TABLE 1. Relative abilities of each of the eight common NTPs to support BVDV NS3 RNA helicase activity[a]

NTP	RNA helicase activity
ATP	1.00
GTP	0.16
CTP	0.31
UTP	0.32
dATP	0.25
dGTP	0.12
dCTP	0.17
TTP	0.28

[a] RNA helicase activity was measured by using the standard RNA substrate under standard reaction conditions as described in Materials and Methods. The indicated NTP was present in the reaction at 5 mM. The extent of strand displacement for each reaction is normalized to that of the reaction containing ATP.

helicase activity preferred or required either a 5′ single-stranded tail, a 3′ single-stranded tail, or no single-stranded region at all in its duplex RNA substrate, the activity of the enzyme was evaluated on three specific RNA substrates. These duplex substrates possessed either 5′ tails only (5′/5′), 3′ tails only (3′/3′), or no single-stranded tails (blunt) (Fig. 1). As shown in Fig. 4A, only the standard RNA substrate and the RNA substrate containing 3′ single-stranded regions (3′/3′) were acted on by the BVDV helicase. No activity was exhibited on the 5′ tailed or blunt-ended substrates. These results are consistent with an RNA helicase that functions in the 3′ to 5′ direction with respect to the template strand.

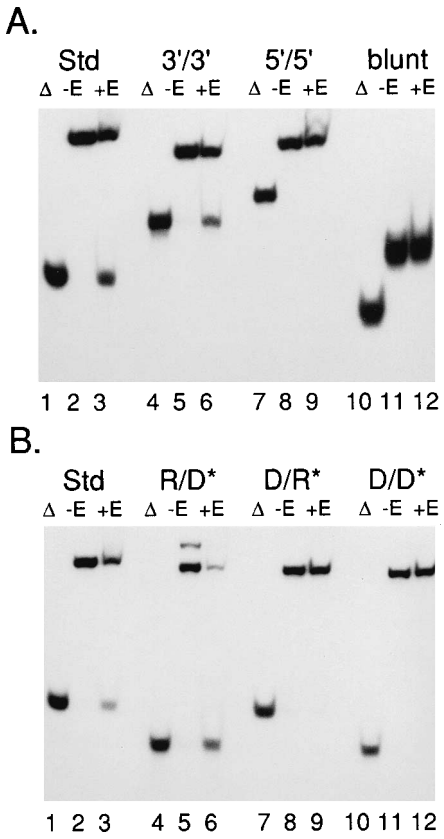

FIG. 4. Substrate specificity of BVDV NS3 helicase. The preparation, structure, and abbreviated designations of helicase substrates are presented in Materials and Methods and Fig. 1. Substrates were incubated in standard reaction mixtures and analyzed by electrophoresis on 15% polyacrylamide gels. (A) RNA substrates. (B) DNA-containing substrates. Lanes: Δ, substrate boiled prior to electrophoresis; −E, native substrate in standard reaction mixture lacking enzyme; +E, complete reaction mixture.

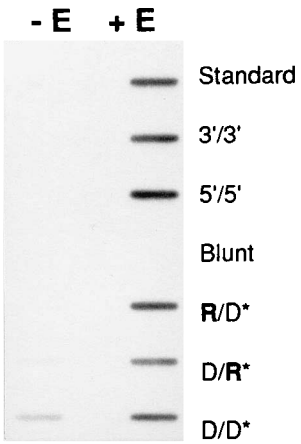

FIG. 5. Filter binding of helicase substrates in the presence of BVDV NS3 helicase. NS3 protein was incubated in standard reaction mixtures lacking ATP with each of the indicated substrates at 37°C for 5 min in a 10-μl volume. Reactions were diluted to 100 μl in a mixture containing 25 mM MOPS-KOH, 2 mM dithiothreitol, and 3 mM $MnCl_2$. Diluted binding reactions were drawn through a nitrocellulose filter using a slot-blot vacuum manifold. Each well was washed by drawing through an additional 300 μl of the above buffer before the filter was air-dried and exposed to X-ray film.

BVDV NS3 helicase activity on DNA-containing duplex substrates. To determine if the BVDV NS3 helicase activity was capable of acting on substrates containing DNA, three duplex substrates were prepared. As depicted in Fig. 1, these DNA-containing substrates were analogous to the standard duplex RNA substrate in structure in that they all possessed both 3′ and 5′ single-stranded regions. They were composed of either an RNA template strand with a DNA release strand (R/D*), a DNA template strand with an RNA release strand (D/R*), or both strands as DNA (D/D*). The NS3 helicase was able to displace a DNA oligonucleotide from an RNA template strand (R/D*) but did not act on the two substrates in which the template strand consisted of DNA (Fig. 4B).

Binding of the BVDV NS3 protein to helicase substrates. The above substrate specificity results suggest that the BVDV helicase might interact only with duplex substrates in which the template strand was RNA. We investigated this possibility by evaluating the interaction of the NS3 protein with ^{32}P-labeled substrate nucleic acids in a nitrocellulose filter binding assay. Each of the seven helicase substrates depicted in Fig. 1 was added to the standard helicase reaction mixture lacking ATP with or without the NS3 protein. In the absence of NS3 protein (but in the presence of bovine serum albumin in the standard reaction mixture) very little or no ^{32}P-labeled substrate bound the filter (Fig. 5, lane − E). However, when NS3 protein was added, all substrates were retained, with the exception of the blunted-ended RNA substrate (Fig. 5, lane + E). The amount of applied substrate retained varied from 50 to 73%, while less than 3% of the blunt substrate bound the filter in the presence of NS3 protein. These filter-binding data indicate that the BVDV NS3 protein binds single-stranded nucleic acid, both RNA and DNA, but does not bind wholly duplex RNA. Similar binding results were obtained in gel mobility shift experiments (data not shown). However, as shown above, the helicase activity is only able to effect strand displacement from substrates in which the template strand is RNA.

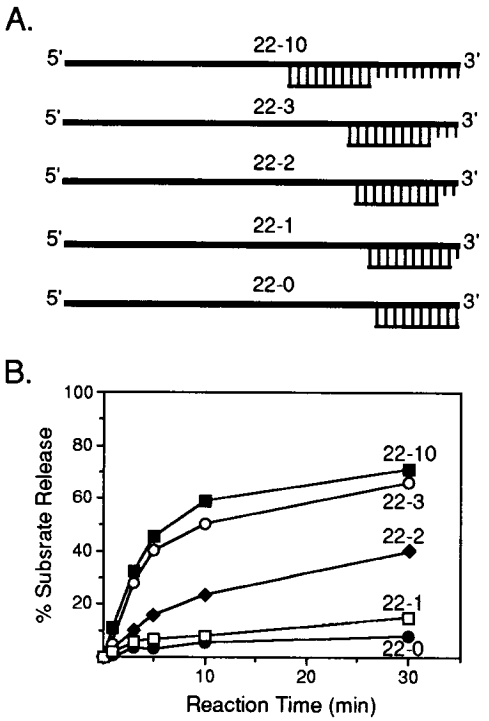

FIG. 6. Requirement of the BVDV NS3 helicase activity for un-base-paired RNA at the 3′ end of the duplex region in helicase substrates. (A) Five helicase substrates, each consisting of the standard RNA template strand annealed with a complementary 22-nt DNA release strand, were prepared as described in Materials and Methods. The DNA oligonucleotides were complementary to the standard RNA template such that they yielded substrates with no free 3′ un-base-paired nucleotides (22-0) or with 1, 2, 3, or 10 un-base-paired nucleotides to the 3′ end of the duplex region (22-1, 22-2, 22-3, and 22-10, respectively). The base composition of oligonucleotides 22-0, 22-1, 22-2, and 22-10 was 45% GC and that of 22-3 was 41%. (B) Each substrate was incubated under standard reaction conditions with the BVDV NS3 enzyme, and helicase activity was determined as a function of reaction time as described in Materials and Methods.

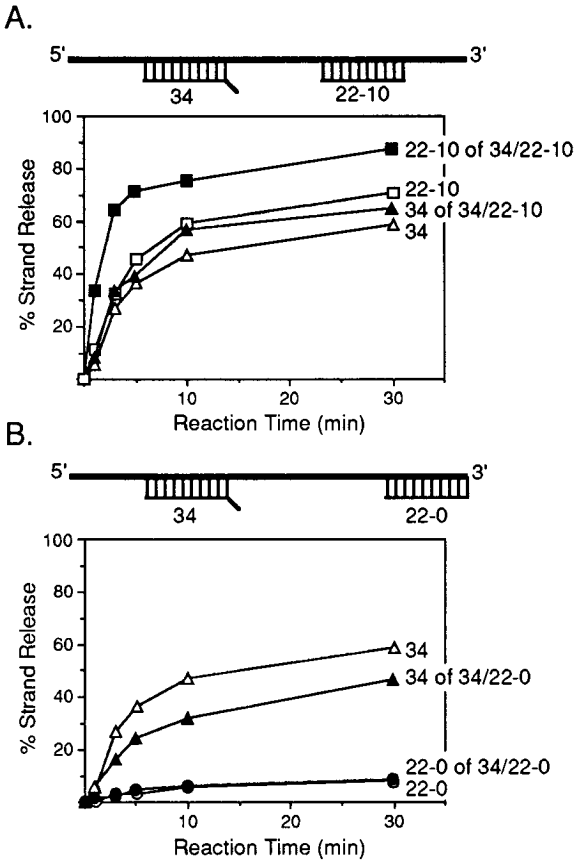

FIG. 7. Ability of BVDV NS3 helicase to act on substrates containing two duplex regions. Two helicase substrates consisting of the standard RNA template strand annealed with two DNA oligonucleotides were constructed. (A) Substrate 34/22-10 represents the 22-10 substrate (Fig. 6) to which oligonucleotide 34 was annealed. Oligonucleotide 34 represents the DNA counterpart to the standard RNA release strand. The distance between oligonucleotides 34 and 22-10 in the template strand was 61 nt. Strand displacement by the BVDV NS3 helicase of each oligonucleotide of the double-hybrid substrate and that of the respective oligonucleotides of each of the single-hybrid substrates (22-10 and 34) was evaluated under standard reaction conditions. (B) Substrate 34/22-0 represents the 22-0 substrate (Fig. 6) to which oligonucleotide 34 was annealed. The distance between oligonucleotides 34 and 22-0 in the template strand was 71 nt. Helicase activity on the double-hybrid substrate and on the two control single-hybrid substrates was determined as described in the legend to panel A.

Requirement for single-stranded RNA 3′ to the duplex region of helicase substrate. Earlier data showed that the BVDV NS3 helicase requires a substrate possessing a 3′ un-base-paired region on the RNA template strand for activity (Fig. 4). To further investigate this requirement, we constructed a series of helicase substrates which possessed various lengths of 3′ un-base-paired regions. Since we found that the BVDV helicase efficiently acted on a substrate in which the release strand was DNA (R/D* substrate, Fig. 4B), we prepared a series of DNA oligonucleotides (22 nt in length) which when annealed to the standard RNA template strand would produce duplex helicase substrates possessing specific lengths of un-base-paired 3′ ends in the template strand. As depicted in Fig. 6A, five substrates had either no free 3′ base (22-0), or 1, 2, 3, or 10 free un-base-paired nucleotides (22-1, 22-2, 22-3, and 22-10, respectively). Results of the evaluation of these duplex molecules as substrates for the BVDV NS3 helicase are shown in Fig. 6B. As expected from earlier data, the 22-0 substrate with its 3′ blunt end was a very poor substrate. Substrate 22-1 was acted on to only to a very limited degree. Strand displacement from substrate 22-2 was significant, but considerably less efficient than that from substrates 22-3 and 22-10. These latter substrates were both equally and efficiently acted on by the NS3 helicase.

Requirement of BVDV NS3 helicase activity for a free 3′ terminus. From the above data, the BVDV NS3 helicase appears to require only three un-base-paired nucleotides to the 3′ end of the duplex region on a helicase substrate for optimal strand displacement. However, with the particular substrates used, it might also be suggested that this minimal requirement further includes a free 3′ terminus in the template RNA. To investigate this possibility, we constructed two substrates, each of which contained two duplex regions. One substrate (34/22-10) represented the 22-10 substrate to which a second DNA oligonucleotide (oligonucleotide 34) was annealed internal to the position of the 22-10 oligonucleotide (Fig. 7A). With this double-hybrid substrate, the NS3 helicase was able to displace both release strands with an efficiency comparable to that seen for each of the respective single-oligonucleotide substrates. The second double-hybrid substrate (34/22-0) consisted of oligonucleotide 34 annealed with the 22-0 oligonucleotide to the standard RNA template strand (Fig. 7B). As seen for the single-hybrid 22-0 substrate, the 22-0 oligonucleotide was not displaced in the double-hybrid substrate (Fig. 7B). However, oligonucleotide 34 was released from the double-hybrid sub-

strate to a level comparable to that seen with the single-hybrid substrate, indicating that the enzyme was able to act on a substrate in which a free 3′ terminus of the template strand was unavailable. Thus, suitable substrates for the BVDV helicase activity do not require a free 3′ terminus but rather only un-base-paired nucleotides on the template RNA to the 3′ end of the duplex region.

DISCUSSION

The NS3 (p80) protein of BVDV is a member of a large family of proteins, the DEAD/DExH helicases, which includes both prokaryotic and eukaryotic cell representatives and numerous virus-encoded polypeptides. These proteins all possess common amino acid sequence motifs that have been associated with NTP binding and hydrolysis activities and with the ability to unwind duplex nucleic acids (8, 10, 14, 21). Proteins in this family participate in a variety of biochemical activities involving both DNA and RNA, which include translation, transcription, splicing, recombination, and replication (30, 38).

For positive-stranded RNA viruses, the putative NTPase-RNA helicase proteins have been subtyped into three groups: alphavirus-like (nsP2-like proteins), picornavirus-like (2C-like proteins), and polypeptides similar to those encoded by the potyvirus-flavivirus-pestivirus groups (NS3-like proteins) (10, 19).

In the alphavirus-like group, the nsP2 protein of Semliki Forest virus has recently been shown to have ATPase and GTPase activities (27). From the picornavirus group, the poliovirus 2C protein also exhibits ATPase and GTPase activities (23, 28). RNA helicase activity has yet to be demonstrated for the NTPase-RNA helicase motif-containing proteins from these virus groups.

Among the third positive-stranded RNA virus group of presumed NTPase-RNA helicase proteins, of which the BVDV NS3 protein studied here is a member, considerable biochemical data are available to substantiate the motif-based predictions of enzymatic activities. RNA-stimulated NTPase activity has been demonstrated for the CI protein of plum pox potyvirus (17), the NS3 protein of the West Nile (39) and yellow fever flaviviruses (37), the NS3 protein of hepatitis C virus (33), and the NS3 (p80) protein of the pestivirus BVDV (34). Regarding the biochemical demonstration of nucleic acid unwinding, Laín et al. (18) showed that the plum pox potyvirus CI protein possessed an RNA helicase activity. Here, we show that the pestivirus BVDV NS3 protein is likewise an RNA helicase. Finally, in data to be presented elsewhere, we have demonstrated that the NS3 proteins of the yellow fever flavivirus and of hepatitis C virus also possess similar helicase activities.

Understanding the specific enzymologic characteristics of these enzymes and their actual functional role in the virus life cycle is a goal of future research. As shown here, the BVDV NS3 RNA helicase requires a substrate possessing a 3′ un-base-paired region on the RNA template strand for activity. This implies that the enzyme binds to the 3′ single-stranded region of the template strand and then, by either a translocation or a cooperative enzyme binding mechanism, progresses toward the duplex segment of the substrate to effect strand displacement. This enzyme appears unable to act in the 5′ to 3′ direction. The directionality of the BVDV NS3 helicase, that is, 3′ to 5′ with respect to the template strand, has been found for other viral RNA helicases, such as the simian virus 40 T antigen (29), the CI protein of plum pox potyvirus (18), and the vaccinia virus NPH-II RNA helicase (32), as well as for cellular enzymes such as human p68 protein (13) and RNA helicase A from HeLa cells (20).

Although the BVDV NS3 protein bound to both RNA- and DNA-containing substrates with single-stranded regions, it was only able to unwind oligonucleotides from an RNA template. This template strand specificity may indicate that the enzyme binds RNA with greater stability, is more efficient at translocation along RNA, or is better able to hydrolyze ATP for strand displacement energy generation when bound to RNA. Additionally, although not fully investigated here, it did appear that the BVDV helicase acted more efficiently to release DNA oligonucleotides from RNA template strands than it did to release RNA oligonucleotides. Further work is necessary to determine the significance of these observations.

Each of the eight common NTPs was able to support strand displacement by the BVDV NS3 helicase on the standard RNA substrate. Consistent with the requirement of NTP hydrolysis for helicase activity, this enzyme had previously been shown to be capable of hydrolyzing all eight NTPs (34). However, since the reaction conditions previously used for measuring NTPase activity and those for the RNA helicase activity described here differ significantly, direct comparison of the efficiency of particular NTP hydrolysis with the RNA helicase activity supported by that NTP is not currently possible and must await a future study.

A common role envisaged for RNA virus RNA helicases is one of causing RNA strand separation during genome transcription and replication. For the BVDV NS3 helicase to release the nascent RNA, it must be supplied with two or three un-base-paired ribonucleotides 3′ of the duplex region on the template strand. During RNA polymerization, this may be provided by a replication bubble at the site of nucleotide addition by the viral RNA replicase. However, events during transcription initiation at the 3′ terminus of the template RNA must not only provide a mechanism for priming nucleotide polymerization by the viral polymerase but, at least for this pestivirus helicase, must also prime strand displacement for the viral helicase activity. The mechanisms of RNA virus transcription initiation and the linkage between RNA polymerization and strand displacement represent exciting areas of future investigation.

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